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Acetylcholinesterase Levels of Aedes Aegypti Larvae after Exposure to the *Pandanus amaryllifolius* Leaf Extracts

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Abstract:- Dengue fever is a disease transmitted by Ae. aegypti. Controlling mosquitoes can use temephos. Temephos larvicides act primarily on the enzyme acetylcholinesterase (AChE) by inhibiting this enzyme. Temephos has drawbacks due to its high risk of resistance, so larvicides from natural ingredients such as pandan leaf extract (P. amaryllifolius) can be an alternative. The mechanism of action of P. amaryllifolius larvicide is still unclear. The purpose of thid study is to ascertain how LC85 pandan leaf methanol extract affects AChE levels in Ae. aegypti. This is a true-experiment design with only a post-test control group research design. Tests were carried out by treating mosquito larvae with P. amaryllifolius LC85 extract, aquades, and temephos for 24 hours and measuring AChE levels with ELISA Reader. The results showed that the AChE enzyme levels of P. amaryllifolius LC85 extract had an average AChE enzyme level of 147.19 + 70.87 units/l. The AChE enzyme levels of larvae exposed to P. amaryllifolius LC85, aquades, and temephos had a significant difference (p <0.05). P. amaryllifolius has potential as a larvicidal, with a mechanism of action as a neurotoxin.

Keywords:- *AChE enzyme; Ae. aegypti; Larvae; Pandanus amaryllifolius.*

I. INTRODUCTION

Dengue fever is a disease transmitted by *Ae. aegypti* and caused by the dengue virus of the genus Flavivirus. This disease can infect all age groups and is found throughout the year [1]. In 2019, 138,127 DHF cases were discovered, an increase from the previous year's total of 65,602 cases. Mortality due to DHF in 2019 is known to have increased from 2018, namely from 467 cases to 919 deaths [2]. Mosquito control can be done in several ways, namely environmental, biological, and chemical control. Chemical control methods include the use of temephos larvicides and fogging. Insecticides are substances used to prevent, damage, repel, or reduce insects, for example, from the organophosphate group. This class of insecticides works primarily on the acetylcholinesterase (AChE) enzyme by inhibiting it [3]. Although effective in eradicating mosquitoes, insecticides have the disadvantage that they can cause resistance [4]. Temephos resistance has been found in Indonesia, namely in the areas of Demak, Banten, and Banjarnegara [5]. One way to deal with temephos resistance is to replace the use of temephos with larvicides made from natural ingredients. Larvicides made from natural ingredients have been proven to be an alternative to reducing mosquito populations so that the number of diseases caused by mosquitoes can be reduced [6].

One of the natural ingredients that can be used as a substitute for temephos and as a natural larvicide is *Pandanus amaryllifolius*, which contains botanical larvicides [7]. *P. amaryllifolius* contains many active compounds such as polyphenols (9.7%), flavonoids (17.18%), saponins (16.4%), and alkaloids (16.6%). Alkaloid compounds work as digestive system poisons and inhibit the AChE enzyme of mosquito larvae. This substance binds irreversibly to the AChE portion and inhibits the enzyme, causing acetylcholine (ACh) to accumulate in the mosquito's synaptic cleft [8]. The potential of these alkaloids is predicted to be realized in this study.

Previous research tested 150 *Ae. aegypti* after 24 hours of exposure to *P. amaryllifolius* extract. Larval mortality was obtained at each concentration of the extract given, with the average percentage of *Ae. aegypti* being highest at a concentration of 50% with a value of 80%. The lowest percentage of larval mortality was at a concentration of 10% with a value of 30% [9]. AChE enzyme activity in *L. acetivum*, which contains mainly alkaloids, has AChE activity at an LC_{50} dose of 0.20 mg/mL. This shows that the alkaloids have an inhibitory effect on the AChE enzyme in insects [10]. Lethal concentration (LC) is the ability of the extract to kill mosquito larvae. LC_{85} is the concentration of the extract needed to kill 85% of mosquito larvae [11]. The purpose of LC_{85} is to maintain the balance of the food chain in the ecosystem. This study used the methanol extract of *P. amaryllifolius* LC_{85}. The purpose of this study was to determine the effect of a methanol extract of *P. amaryllifolius* LC_{85} on levels of the AChE enzyme in *Ae. aegypti*.

II. METHODS

This study employs a pure experimental research design (true-experimental design) with a post-test only control group research. This research has been declared ethically feasible with letter number No. 008/EC/KEPK-FKUC/VII/2022. The materials used in this study were *P. amaryllifolius*, methanol, coarse filter paper, tween 20, aquades and QuantiChromTM Acetylcholin esterase Assay Kit, which contains an assay buffer, reagent and calibrator, gloves, masks, temephos, aquades, DMSO, Tris buffer pH 7.8, acetylcholine iodide solution (ATCI), DTNB solution (5,5 -dithiobis-2-nitrobenzoic acid, Ellman reagent), and

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three tips [blue tip, yellow tip, white tip]. The tools used are glass jars, funnels, 1000 ml Erlenmeyer, 500 ml beaker, rotary evaporator, spatula, analytical balance, plastic cup, gauze, thermometer, litmus paper, psychrometer, handcounter, microplate 96 wells, ELISA reader, container transparent sample (cuvette), and micropipette.

The determination of the *P. amaryllifolius* species was carried out at the Faculty of Pharmacy, Widya Mandala University, Surabaya, to officially ensure that the species was *P. amaryllifolius*. The *P. amaryllifolius* used was collected from Made, Sambikerep District, Surabaya City, East Java (7°16' 33.7" S 112°38' 42.6" E). In the process of making the extract, the leaves are cut and dried for a month. After drying, the leaves are crushed into powder (simplicia) and soaked in methanol (maceration) for 2 weeks. The maceration results were filtered using a funnel and filter paper and evaporated using a rotary evaporator to obtain the extract.

Preliminary tests were carried out first to ensure the correct concentration of the extract used was LC_{85}, using several doses. After obtaining the right concentration, the actual test is carried out. The mother liquor is prepared using the following formula:

$$A\ ppm = \frac{A\ mg\ extract}{1000\ ml\ aquades}$$

After that, the main solution of the methanol extract of *P. amaryllifolius* was diluted into several concentrations using the dilution formula:

$$V1 \times N1 = V2 \times N2$$

Information
V1 = volume of mother liquor
N1 = concentration of mother liquor
V2 = desired volume
N2 = desired concentration

The biolarvicidal test was carried out at the Entomology Laboratory of the Institute of Tropical Diseases, Airlangga University, Surabaya. The mosquito larvae of *Ae. aegypti* as many as 20 larvae were put into a clear glass, and each of them would be given a different treatment. Container 1 will be given aquades (a negative control), container 2 will be given temephos (a positive control), and container 3 will be given *P. amaryllifolius* LC_{85} extract. The treatment was left for 24 hours, and after that. the enzymes were measured in the larvae.

An AChE enzyme examination was carried out at the Laboratory of Professor Nidom Foundation in Surabaya. Five dead mosquito larvae were taken randomly from each treatment, and the AChE enzyme levels were measured using the QuantiChrom™ Acetylcholin Esterase Assay Kit. The mosquito larvae were crushed individually and made into a homogenate, which was dissolved in 0.5 ml of 0.1 M phosphate buffered saline (PBS) solution, with a pH of 7.5 [3]. The homogenate was then centrifuged at 14,000 rpm for 5 minutes, and the supernatant was obtained, which was transferred to the microplate using a micro pipette. The reagent was prepared by adding 200 µl of assay buffer to 2 mg of reagent and then vortexing it until it dissolved. In each well with the supernatant on the microplate, 190µl of the reagent mixture was added. The microplate is inserted into the ELISA reader, and the optical density (OD), or what can be called the adsorption, is read at a wavelength of 405 nm in the first 5 minutes. The percentage of AChE inhibition can be calculated based on the adsorption value using the following formula [12]:

$$\%\ \text{Inhibition} = \left(1 - \frac{A_t}{A_0}\right) \times 100$$

Information:
A0: Adsorbance control
At: The adsorption of the compound tested

AChE enzyme levels are expressed as Mean ± Standard Deviation (SD) and visualized in graphical form. The data that has been obtained will be tested for normality first. If p> 0.05 then the data is normally distributed. Then the data is tested for homogeneity (if sig. > 0.05 then the data is homogeneous). If normal distribution data is obtained, the one-way ANOVA test will be continued, and if data with an abnormal distribution is obtained, the Kruskal Wallis test will be carried out. After that, the Post hoc test is then repeated, with the Tukey/Duncan test used if the data is homogeneous and the Mann Whitney test used if it is not.

III. RESULTS

In the preliminary test, it was found that the concentration of the extract needed to kill 85% of mosquito larvae was 35.000 ppm. For complete preliminary test results, see the following table:

Table 1: Preliminary Test Result

P. amaryllifolius concentrations	Replication	Mortality
1.000 ppm	1	8
	2	6
5.000 ppm	1	12
	2	12
8.000 ppm	1	15
	2	18
12.000 ppm	1	15
	2	19
15.000 ppm	1	17
	2	16
35.000 ppm	1	18
	2	17

In the biolarvicidal test (the real test), the mortality of *Ae. aegypti* is as follows:

Table 2: The Number of Mortality of *Ae. aegypti*

Treatment type	Replication	Mortality of *Ae. aegypti* larvae	
		Average (amount)	Average (%)
P. amaryllifolius	1	17	85%
	2	16	80%
	3	18	90%
	1	0	0%
Aquades	2	0	0%
	3	0	0%
	1	20	100%
Temephos	2	20	100%
	3	20	100%

This research will be analyzed regarding the effects of the methanol solution of *P. amaryllifolius* LC_{85} on AChE levels in *Ae. aegypti* instar III. The first step is to describe the results of AChE enzyme levels (units/l) using descriptive statistics. The following table shows the characteristic results of AChE enzyme levels in each treatment:

Table 3: Characteristics AChE enzyme levels (unit/l)

Groups	Min	Max	Median	(Mean ± SD)
P. amaryllifolius	86,58	225,11	129,87	147,19 ± 70,87
Aquades	294,37	363,64	311,69	323,23 ± 36,05
Temephos	277,06	285,71	277,06	279,94 ± 4,99

A normality test for AChE enzyme level data was carried out to find out whether the research data was normally distributed or not. The normality of the data will be checked using the Shapiro Wilk test. In this study, a significant level of 5% will be used; if a p-value > 0.05 is obtained, it can be concluded that the data has been normally distributed. The normality test results can be seen as follows:

Table 4: AChE Enzyme Level Normality Test Results

Groups	*p*	Description
P. amaryllifolius LC_{85}	0,593	Normal
Aquades	0,463	Normal
Temephos	0,000	Abnormal

P > 0,05 (Normally distributed)

The homogeneity test on the AChE enzyme level data was carried out to find out whether the research data had a homogeneous variance or not. At a significant level of 5%, if a p-value > 0.05 is obtained, the data already has a homogeneous diversity of variance values. The results of the data homogeneity test for AChE enzyme levels can be seen in the following table:

Table 5: Results of Homogenity Test of AchE Enzyme Levels

Groups	*p*	Description
P. amaryllifolius LC_{85} Aquades Temephos	0,067	Homogen

P > 0,05 (Homogen)

This test shows that from the two tests, the assumptions of the normal distribution test are not fulfilled and the homogeneity test is fulfilled, so the next test to test for a significant difference in AChE enzyme levels will be the Kruskall Wallis test followed by a post hoc test using the Mann Whitney test to find out which sample had the most significantly different AChE enzyme levels.

The Kruskall Wallis test is a statistical test to determine significant differences in AChE enzyme levels in different solutions. At a significant level of 5%, if the p-value <0.05, it means that there is a significant difference between the extract solutions, if the p-value is > 0.05, it means that there is no significant difference between the solutions. The following table shows the results of the Kruskall Wallis test.

Table 6: Kruskall Wallis Test Results AChE Enzyme Levels (unit/l)

Groups	Median	(Mean ± SD)	*p*	Description
Pandan LC_{85}	129,87	147,19 ± 70,87		
Aquades	311,69	323,23 ± 36,05	0,027	Significantly
Temephos	277,06	279,94 ± 4,99		different

Description: (*) = Significantly different at a significant level of 5% ($p < 0,05$)

The results of the statistical tests showed that there were significant differences in the levels of the AChE enzyme in each group of pandan LC_{85}, aquades, and temephos solutions. The post hoc test with the Mann Whitney test was used to find out which solution had the highest levels of the AChE enzyme and had a significantly different value from the other solutions.

Tabel 7: Post-Hoc Test Results (Mann Whitney) AChE Enzyme Levels (unit/l)

Groups	Pandan LC_{85}	Aquades	Temephos
P. amaryllifolius LC_{85}			
Aquades	0,050*		
Temephos	0,046*	0,046*	

Description: Sign (*) = Significantly different at a significant level of 5% (p < 0,05)

Post-hoc test results (Mann Whitney) the AChE enzyme levels of larvae exposed to P. amaryllifolius LC85, aquades, and temephos had a significant difference (p <0.05). Based on table 5, the median value of P. amaryllifolius LC85 was 129.87 units/l, aquades 311.69 units/l and temephos 277.06 units/l. This indicated that treatment with P. amaryllifolius LC85 had the lowest levels of the enzyme AChE and was significantly different from the aquadest and temephos groups. While the group with the highest enzyme levels was aquades.

The following is an illustration of the average AChE enzyme levels in each treatment, which can be seen in the following figure.

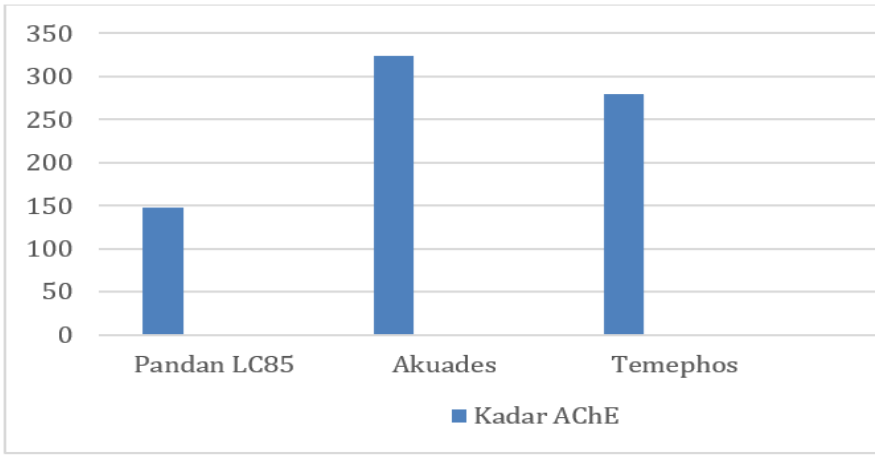

Fig. 1: Diagram of Average AChE Enzyme Levels in Each Group

IV. DISCUSSION

P. amaryllifolius has an influence on the mortality of *Ae. aegypti* at a concentration of 35,000 ppm, which is 85%. This ability is similar to research on the effect of *P. amaryllifolius* at a concentration of 200 ppm, which will kill an average of 25 (100%) *Aedes sp.* larvae and is proven to be used as a larvicidal mosquito, *Ae. aegypti*. The use of solvents in extraction will affect the quality of the extract and the compound content in plant extracts because ethanol will function as a solvent and will pull out the active compounds in the extract [13]. This is in accordance with research that examines the differences in the content of extracts with ethanol solvents and without solvents (water). This study showed that extracts with ethanol solvents contained alkaloids, flavonoids, saponins, tannins, and phenols, while extracts without solvents (water) contained flavonoids, tannins, and phenols [14].

P. amaryllifolius toxicity was also found in *Musca domestica*, with the lowest mortality of one fly (5%) and the highest mortality of 13 flies (15%). This study showed that *P. amaryllifolius* extract was toxic to insects [15]. In addition, the toxicity ability of *P. amaryllifolius* to *Anopheles* sp. at a concentration of 1,000 ppm, an average death rate of 19 larvae (94%) and at a concentration of 400 ppm, an average death rate of 14 larvae (60%) [16].

Apart from *P. amaryllifolius*, there are several other types of *Pandanus* that contain chemical compounds similar to *P. amaryllifolius*. In previous studies, it was found that the content of alkaloids, steroids, phenols, tannins, terpenes, flavonoids, saponins, and glycosides in *P. tectorius* extract [17]. The content of secondary metabolites in red fruit (*Pandanus conodieus Lam.)* was determined by extraction using n-hexane, methanol, ethyl acetate, and water solvent, although different solvents were obtained, all samples

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contained flavonoids, terpenoids, and alkaloids, except for samples with n-hexane solvent [18]. The *Pandanus* genus, such as *P. amaryllifolius*, *P. dubius*, and *P. utility*, are sources of secondary metabolites, namely steroids, terpenoids, flavonoids, lignans, benzenoids, and alkloids [19].

AChE is a hydrolytic enzyme that functions in cholinergic nerve transmission by hydrolyzing acetylcholine (ACh) into acetate and choline. Organophosphate larvicides will act on the AChE enzyme, which has a mechanism of action to bind the amino acid serine permanently. AChE, which is inhibited, will increase muscle impulses so that they will contract continuously, muscle spasms occur and end in insect death [20]. Temephos will enter the larvae through the exoskeleton through the tarsus, and its mechanism of action involves inhibiting the AChE enzyme so that ACh buildup will occur in the nerve endings, which will cause hyperexcitation, seizures, muscle paralysis, and death [21].

P. amaryllifolius is known to contain polyphenolic compounds, flavonoids, saponins, essential oils, tannins, and alkaloids [22]. One of these compounds, namely alkaloids, is a neurotoxin that works by inhibiting the AChE enzyme so that it cannot hydrolyze ACh into acetate and choline. This inhibition causes ACh to accumulate in the nerve endings, increasing the work of the larval body in constantly sending commands to the larval muscles, causing the muscles contract continuously and be difficult to control [23].

The AChE enzyme has an important function at nerve synapses. AChE levels of *Ae. aegypti* and *Ae. albopictus* after exposure to *Bryopsis pennata*, *Padina australis*, and *Sargassum binderi* at LC_{50} was between 1-10 mg/ml. AChE levels of larvae after exposure to *Sargassum angustifolium* LC_{50} were 5.4 mg/ml, *Sargassum boveanum* LC_{50} were 1.0 mg/ml, *Sargassum oligocystum* LC_{50} were 2.5 mg/ml and *Sargassum Sp.* from India LC_{50} of 1.0 mg/ml. AChE levels after exposure to *P. australis* LC_{50} showed better results, namely 6.3 mg/ml [24]. AChE activity in mosquito larvae exposed to temephos was 70% and 60%. This shows that inhibition of AChE will cause AChE activity to decrease and cause the death of the larvae [25]. Reduced levels of the enzyme AChE appear to be an inhibition of the enzyme's synthesis. This requires further research at the molecular and protein (proteomic) levels. In this study, the AChE levels of mosquito larvae exposed to *P. amaryllifolius* LC_{85} extract had an average AChE enzyme level of 147.19 + 70.87 units/l with the lowest level being 86.58 units/l and the highest level being 225.11 units/l.

The mechanism of action of the alkaloids is that when they enter the body of the larvae through the mouth and skin, the alkaloids inhibit the AChE enzyme, disrupt endocrine functions by interfering with the molting function, and are toxic to nerves. AChE enzyme inhibition will lead to accumulation of ACh at the synapses and trigger constant excitation of the larvae, ataxia, and a lack of muscular coordination until it ends with the death of the larvae [26].

V. CONCLUSION

- There is an effect of the methanol extract of P. amaryllifolius LC85 on the levels of the acetylcholinesterase enzyme.
- AChE enzyme levels of P. amaryllifolius LC85 extract had an average AChE enzyme level of 147.19 + 70.87 units/l with the lowest level being 86.58 units/l and the highest level being 225.11 units/l.
- There were significant differences in the levels of the AChE enzyme in each group of pandanus LC85 solution, aquades, and temephos.

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